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Gustatory neurons derived from epibranchial placodes are attracted to, and trophically supported by, taste bud-bearing endoderm in vitro

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Abstract

Taste buds are multicellular receptor organs innervated by the VIIth, IXth, and Xth cranial nerves. In most vertebrates, taste buds differentiate after nerve fibers have reached the lingual epithelium, suggesting that nerves induce taste buds. However, under experimental conditions, taste buds of amphibians develop independently of innervation. Thus, rather than being induced by nerves, the developing taste periphery likely regulates ingrowing nerve fibers. To test this idea, we devised a culture approach using axolotl embryos. Gustatory neurons were generated from cultured epibranchial placodes, and when cultured alone, axon outgrowth was random over 4 days, a time period coincident with axon growth to the periphery in vivo. In contrast, cocultures of placodal neurons with oropharyngeal endoderm (OPE), the normal taste bud-containing target for these neurons, resulted in neurite growth toward the target tissue. Unexpectedly, placodal neurons also grew toward flank ectoderm (FE), which these neurons do not encounter in vivo. To compare further the impact of OPE and FE explants on gustatory neurons, cocultures were extended and examined at 6, 8, and 10 days, when, in vivo, placodal fibers have innervated the epithelium but prior to taste bud formation, when taste buds have differentiated and are innervated, and when the mouth has opened and larvae have begun to feed, respectively. The behavior of placodal axons with respect to target type did not differ between OPE and FE cocultures at 6 days. However, by 8 days, differences in axonal outgrowth were observed with respect to target type, and these differences were enhanced by 10 days in vitro. Most clearly, exuberant placodal fibers grew in 10-day OPE cocultures, and numerous neurites had invaded OPE explants by this time, whereas gustatory neurites were sparse in FE cocultures, and rarely approached and almost never contacted FE explants. Thus, embryonic endoderm destined to give rise to taste buds specifically attracts its innervation early in development, as placodal neurons send out axons. Later, when gustatory axons synapse with differentiated taste buds in vivo, the OPE provides trophic support for cultured gustatory neurons.

Introduction

In all vertebrates, taste buds are innervated by branches of the VIIth, IXth, and Xth cranial nerves (Northcutt et al., 2000; Smith and Davis, 2000). The neurons of these cranial ganglia have dual origins—arising from both neural crest and epibranchial placodes (Narayanan and Narayanan, 1980), but those derived from placodes are thought to innervate predominantly taste buds, as well as other viscerosensory targets (Landacre, 1910, 1933; Webb and Noden, 1993; Graham and Begbie, 2000; Baker and Bronner-Fraser, 2001). In contrast, taste buds, which comprise excitable, neuron-like cells, do not derive from neurogenic ectoderm, but rather arise directly from local oral and pharyngeal epithelia (Barlow and Northcutt, 1995; Stone et al., 1995). Thus, these two cell populations, sensory neurons and taste buds, develop separately but must connect later during embryogenesis to form a functional taste system at birth.

In mammals, circumstantial evidence suggests that the developing taste periphery provides guidance cues for afferent taste fibers. Mammalian taste buds are housed in epithelial specializations or papillae, which develop in the lingual epithelium before taste buds differentiate (Mistretta, 1972). Development of taste papillae and taste bud primor-

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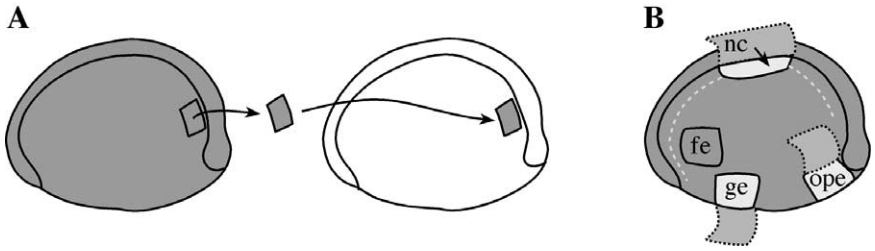

Fig. 1. Schematic diagrams of embryonic regions used in the grafting and coculture experiments. (A) Cranial ectoderm immediately lateral to the neural folds containing presumptive epibranchial placodes was removed from the right side of stage 19 pigmented axolotl embryos, and grafted isotopically and isochronically into albino hosts to determine whether preplacodal ectoderm will contribute sensory neurons to appropriate cranial nerve ganglia. (B) Presumptive epibranchial placodal ectoderm was explanted and placed in culture alone, or with four different targets: oropharyngeal endoderm (OPE), flank ectoderm (FE), gut endoderm (GE), or notochord (NC). The embryo depicted here illustrates the location of each of the embryonic regions used as targets in coculture experiments, Anterior is to the right.

dia are initially nerve-independent, in that these structures begin to develop prior to arrival of nerve fibers (Hall et al., 1999; Mbiene and Roberts, 2003). Further, papillae will develop in isolated tongue cultures devoid of innervation (Farbman and Mbiene, 1991; Mbiene et al., 1997; Nosrat et al., 2001; Hall et al., 2003; Mistretta et al., 2003). In amphibians, while differentiated taste buds are not found until after the epithelium is innervated, taste buds will form in the complete absence of nerves (Barlow et al., 1996; Barlow and Northcutt, 1997). Thus, in both mammals and amphibians, the taste periphery initially develops autonomously and likely dictates the subsequent development of its own innervation.

In many other developing neural systems, axon guidance is accomplished through the combined and/or sequential influences of both attractive and repulsive cues, and these guidance cues comprise both contact-dependent and/or short range signals, as well as diffusible, longer range signals (Goodman and Tessier-Lavigne, 1997; Tessier-Lavigne and Goodman, 1996). To test whether guidance factors emitted by target tissue attract or repel gustatory afferents during embryonic development, we first generated an embryonically homogeneous pool of presumed gustatory neurons, by isolating and culturing ectoderm containing presumptive epibranchial placodes from axolotl embryos (Northcutt and Brändle, 1995; Stone, 1922). These preplacodal ectoderm explants give rise to neurons, which exhibit sustained axonal outgrowth in vitro. The question of axon guidance was then tested by pairing presumptive placodal ectoderm with a number of potential target tissues in vitro, including the appropriate target oropharyngeal endoderm, destined to give rise later to taste buds (Barlow and Northcutt, 1995), as well as several inappropriate targets not normally innervated by these placodal neurons. We show here that early, long-range, diffusible cues emitted by oropharyngeal endoderm guide sensory afferents in vitro, and that these same cues may be present in flank ectoderm. Placodal axons also exhibit inherent directional specificity, in that only tropically active targets placed to the anterior of placodal explants are attractive. Further, in long-term cultures, gustatory neurons appear to be supported trophically by signals unique to the oropharyngeal endoderm, and these factors are not present in flank ectoderm. Interestingly, much earlier in development, oropharyngeal endoderm induces epibranchial placode neurogenesis (chick: Begbie et al., 1999; axolotls: S. Matz and R.G. Northcutt, personal communication). Thus, our findings extend the context of the signaling interactions between placodal ectoderm and oropharyngeal endoderm, from early induction, to later axon guidance and neurotrophic support.

Materials and methods

Ambystoma mexicanum embryos were acquired from the Indiana University Axolotl Colony (Bloomington, IN) and maintained in 20% Holtfreter's solution at 22°C.

Transplant surgery

Embryos were staged according to Bordzilovskaya et al., (1989). The ectoderm containing the presumptive epibranchial placodes was located in stage 19 axolotl embryos by using previously defined landmarks and diagrams (Fig. 1A; Stone, 1922). The presumptive epibranchial placodes can be removed with the ectoderm immediately lateral to the neural folds without including the dorsolateral set of placodes, which reside within the lateral walls of the neural folds at this stage (Northcutt et al., 1996). Embryos were stabilized in wells in a plasticine-lined petri dish while immersed in 100% Holtfreter's solution (HF) supplemented with 400 mg/L penicillin, 400 mg/L streptomycin, and 20 mg/L gentamycin (pH 7.6). The presumptive placodal ectoderm was removed from pigmented donor embryos with flame-etched tungsten needles and grafted orthotopically into albino hosts (Fig. 1A).

Culture of placodal ectoderm with and without target tissue

Presumptive placodal ectoderm (Fig. 1A) from stage 19 embryos was placed in culture either alone or with potential

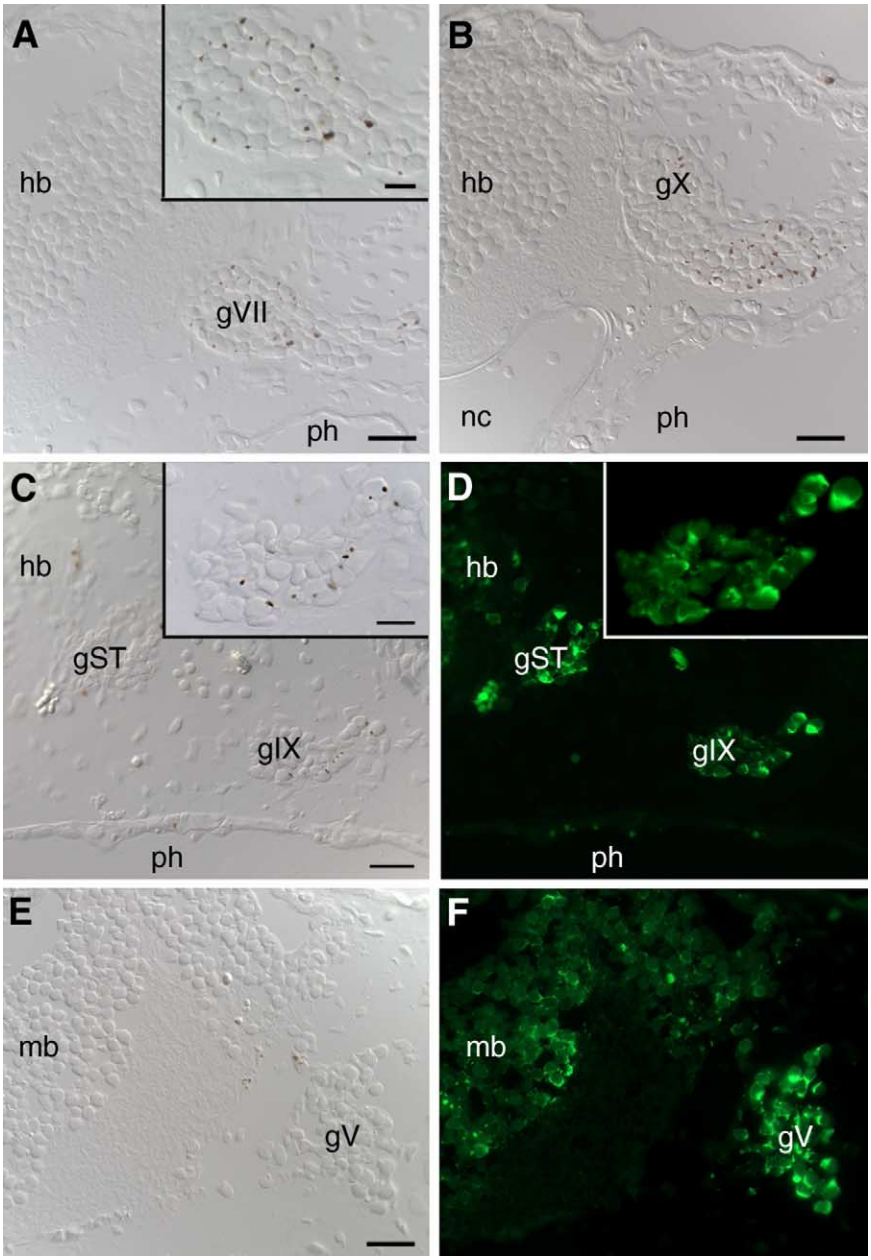

Fig. 2. Presumptive epibranchial placodes contribute sensory neurons to the ganglia of VIIth, IXth, and Xth cranial nerves, but not to trigeminal (Vth) or lateral line ganglia. Bright field micrographs of cryosections of stage 41 albino larvae that received pigmented ectodermal grafts at stage 19 reveal the distribution of donor cells containing pigment granules (A–C, E). (D, F) The sections shown in (C) and (E) were also immunostained with the anti-Hu antibody (green), which selectively labels neuronal cell bodies. Neurons in the VIIth (A), IXth (C, D), and Xth (B), but not the Vth (E, F) cranial nerve ganglia, possessed pigment granules. Insets in (A), (C), and (D) are high magnification views of the ganglia in each panel, to best demonstrate the distribution of pigment granules within cranial ganglion cells (in A and C), as well as the neuronal identity of these cells (anti-Hu staining in D). gV, Vth cranial nerve ganglion; gVII, Vth cranial nerve ganglion; gIX, IXth cranial nerve ganglion; gX, Xth cranial nerve ganglion; gST, supratemporal lateral line ganglion; hb, hindbrain; mb, midbrain; nc, notochord; ph, pharynx. Scale bars in (A–F), 50 μm; insets in (A) and (C), 20 μm.

target tissues also taken at stage 19. Four different targets were tested (Fig. 1B): (1) Oropharyngeal endoderm (OPE) was obtained by removing the overlying ectoderm and cutting out the endoderm posterior to the prosencephalon and anterior to the heart field (Barlow and Northcutt, 1997). (2) Flank ectoderm (FE) was removed from the posterolateral trunk. (3) Notochord (NC) was obtained via a ventral approach, which entailed removal of the gut. (4) Gut endoderm (GE) was taken from a midventral section of the gut, after the overlying ectoderm was removed.

All explants were cultured in Growth Factor Reduced (GFR) Matrigel (Becton Dickinson). GFR Matrigel was diluted 1:1 with 60% Leibowitz'-15 medium (L-15; Sigma) supplemented with 400 mg/L each of penicillin and streptomycin, and 25 mg/L gentamycin (pH 7.6). A 35- to 40-μl bed of cold GFR Matrigel was placed on a sterile, acid-

Table 1
Timing of placodal neuron development in axolotls at 22°C

	Removal of presumptive placodes (this study)	All epibranchial placodes are present	Cranial nerve fibers reach the oropharyngeal epithelium	Taste buds are differentiated and innervated	Feeding begins
Stage in vivo[a]	19	28[b]	37/8[c]	41[c]	43+
Day in vitro	0	1	4	8	10

[a] Bordzilovskaya et al., 1989.
[b] Northcutt and Brändle, 1995.
[c] Barlow et al., 1996.

washed coverglass in a sterile 35-mm plastic petri dish, which was then incubated at 37°C in a humidified chamber for 30 min to allow the gel to polymerize. Next, 40–45 μl of cold gel was placed over the solidified gel bed. Target tissues were explanted up to 30 min before being placed in the gel, while placodal ectoderm was explanted just prior to embedding in GFR Matrigel. Placodal explants were oriented with anterior to the right and were placed either alone (controls) or at 200 μm from a target explant. All cultures were placed again at 37°C for 30 min to polymerize the gel dome. Finally, the cultures were flooded with 4 ml of 60% L-15 medium with 1% bovine serum albumin (BSA, Sigma), antibiotics, and antimycotic, and allowed to develop at 22°C for 4, 6, 8, or 10 days. The initial distance of 200 μm between placodal ectoderm and targets occasionally shifted during the culture period. Only cocultures with final interexplant distances of 150 to 700 μm were analyzed. Cocultures with interexplant distances outside this range were discarded.

Immunofluorescence

Embryos with placodal ectoderm grafts

Embryos with placodal grafts were fixed at stage 41 (hatching) in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. After rinsing in PBS, larval heads were removed and placed in 10% sucrose in PBS for 20 min, prior to embedding in OCT compound (Tissue Tek). Frozen OCT blocks were cryosectioned at 10–16 μm. For immunofluorescence, sections were processed via standard methods (Barlow et al., 1996; Barlow and Northcutt, 1997). The primary antibodies used were mouse anti-acetylated alpha tubulin, at 1:1000 (anti-AT, Piperno and Fuller, 1985; Sigma) and mouse anti-Hu, at 1:500 (Molecular Probes; Eugene, OR) in PBST (PBS with 0.03% Triton X-100), overnight at 4°C. After rinsing, sections were incubated in either goat anti-mouse IgG2B conjugated with TRITC at 1:500 (Southern Biotechnologies, Inc.) or goat anti-mouse Alexa 546 at 1:1000 (Molecular Probes) in PBST overnight at 4°C. Sections were rinsed and counterstained with Hoechst 33243, at 1:10,000 to label nuclei (Molecular Probes), and coverslipped with Fluoromount G (Southern Biotechnologies, Inc.).

Explant cultures

Cultures were fixed in cold 4% paraformaldehyde for 1 h. Cultures were then washed four times in PBS, and either processed immediately for immunofluorescence or stored in sterile PBS with 0.001% sodium azide to retard bacterial growth. Cultures were processed in whole mount with anti-AT to detect nerve fibers. The protocol is identical

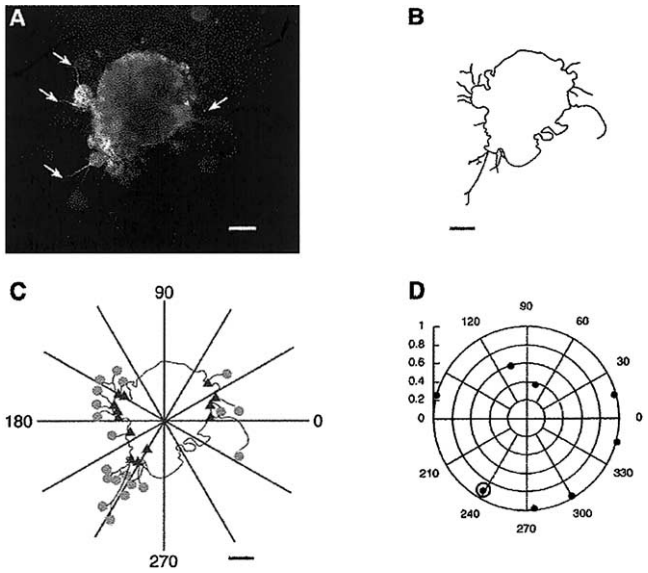

Fig. 3. Assessment of neurite outgrowth from cultured epibranchial placodal ectoderm. Ectoderm was explanted at stage 19, prior to the formation of epibranchial placodes, and cultured for 4 days. (A) Fixed 4 day cultures were immunostained in whole mount with antibodies to acetylated alpha-tubulin to detect neurites (white arrows). (B) Immunostained explants and nerve fibers were traced using a camera lucida. Note that many more fibers are evident in the drawing, since it includes fiber distribution in all focal planes, whereas (A) is a confocal image of a single optical section. (C) Fiber tips (gray circles) and fibers exit points (black triangles) were identified and quantified, based on the orientation of the ectodermal explant. 0°/360° marks the anterior of the explant, while 90° is dorsal, 180° is posterior, and 270° is ventral. (D) The mean angle of outgrowth was determined by using first order analysis of the polar data (see methods for details). The mean angles of eight explants were graphed on a polar plot with the orientation of the placodal explant as in (C), and a secondary analysis applied to the data (see materials and methods for details) to determine whether growth was directed or random when placodal ectoderm was cultured alone. In the case of these control explants, fiber was growth was random. The mean angle for the explant in (A–C) is circled in black. Scale bar, 100 μm.

to that for sections, including the use of the secondary goat anti-mouse IgG2B TRITC at 1:500 (Southern Biotechnologies, Inc.).

Image acquisition

High-resolution digital images were obtained with a Zeiss Axioplan fluorescence microscope, and with either a black and white, cooled CCD camera (ORCA, Hamamatsu) using Openlab software (Improvision, UK), or a Zeiss high resolution Axiocam CCD camera with Axiovision software. Images were saved as TIFF files, pseudo-colored if necessary, contrast adjusted, and multichannel images merged in Adobe Photoshop 6.0 for Macintosh.

Immunostained cultured explants in whole mount were traced with a camera lucida to document axon outgrowth from placodal explants and the relative locations of target tissue and placodal ectoderm explants.

Quantitative analysis

Several measures of neurite outgrowth were acquired for cultures at 4, 6, 8, and 10 days in vitro, including the mean and standard error for: (1) the number of fibers exiting from each explant; (2) the number of fiber tips per culture; (3) the length of the five longest fibers per culture (only for placodes grown alone); and for older cultures, (4) the number of fibers contacting target explants. To obtain the length data, five or more of the longest fibers were selected by eye from camera lucida drawings. Each fiber was measured by using a length of dental floss, which was then stretched taut and converted to μm via comparison with a calibration mark on a drawing made from a stage micrometer.

Polar plot data of the distribution of axon outgrowth within individual cultures were generated in two ways. The first was to map the exit points of fibers from the explant onto a 360° plot, with 0/360° marking the anterior of the explant, 90° marking the dorsal region of the explant, etc. The second approach entailed plotting the circular distribution of all fibers tips distant from the explant. To do this, the center of the placodal explant was estimated by using a compass-drawn circle around the entire explant. Circular distribution data were acquired only for 4-day-old cultures.

Statistical tests

First order analysis of the circular plot data generated a mean angle for the direction of fiber growth, as well as an *r*-value, which is a measure of the degree to which fibers are collected around the mean angle. Second order analysis of these data allowed determination of the collective mean angle of fiber growth and *r*-value for a group of cultures within a treatment. From these analyses, we could determine whether placodal fiber growth was directed or random when placodal neurons were grown alone or with various targets.

Table 2
Summary of gustatory neurite outgrowth in vitro at 22°C

Day in vitro (Stage in vivo)	Number of explants (*n*)	Number of exiting fibers (x ± se)	Number of fiber tips (x ± se)	Five longest fibers (μm) (x ± se)
4 (37/8)	18	8.4 ± 3.4	18.3 ± 4.4	206.4 ± 31.8
6 (39)	22	29.7 ± 5.4	44.7 ± 7.6	506.6 ± 52.3
8 (41)	18	37.6 ± 6.1	59.4 ± 9.5	895.8 ± 70.6

First order tests for circular distribution data (Zar, 1999)

A first order analysis allowed us to determine both the mean angle of fiber growth within a single culture and the degree to which that growth was clustered or dispersed around the mean angle. Calculating the *r* value (*r* = the degree to which fibers are collected around a mean angle): *fi* = number of fibers within a given arc bin; *ai* = a specific arc bin, e.g., 0° to 30°; *n* = total number of fibers that grew out of a placodal explant.

$$X = \frac{\sum fi \cos ai}{n}$$

$\Sigma fi \cos ai$ = summed value of the number of fibers within a given arc value multiplied by the cosine of that arc value.

X = the above value divided by the number of arc bins around our circle (i.e., 12);

$$Y = \frac{\sum fi \sin ai}{n}$$

$\Sigma fi \sin ai$ = the summed value of the number of fibers within a given arc value multiplied by the sine of that arc value.

Y = the above value divided by the number of arc bins around our circle (i.e., 12).

$$r = \sqrt{X^2 + Y^2}$$

r is a measure of collection of values around a mean angle, and ranges from 0.0 to 1.0. A large value indicates fibers are collected around the mean angle, whereas a small value signifies fibers are dispersed around the mean angle.

Calculating the mean angle:

$$\cos \bar{a} = \frac{X}{r}$$

$\bar{a}$ = mean angle of one explant, determined by dividing X (see above) by *r*, the measure of fiber collection.

Second order tests

A second order analysis was used to determine the mean of a group of mean angles of explants within a treatment. This test allows for the calculation of the mean angle for a set of explant cultures ($n = 8$, all treatments).

$$Xj = rj \cos \bar{a}j$$

Xj = the X value for a single explant culture (denoted *j*). It

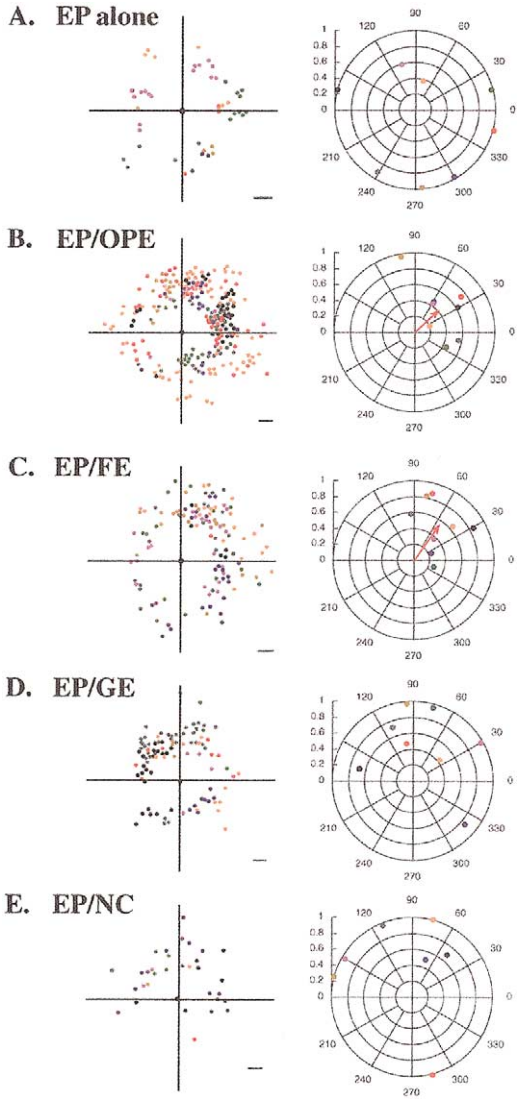

Fig. 4. In short term cocultures, gustatory fibers from epibranchial placode explants (EP) are directed toward oropharyngeal endoderm (OPE) or flank ectoderm (FE), but grow randomly in the presence of gut endoderm (GE) or notochord (NC). Scattergrams of the distribution of fiber tips around each of eight placodal explants for each type of coculture are on the left, while the distribution of the mean angle of fiber outgrowth for each of the eight cultures is depicted by the colored dots on polar plots on the right. Each color represents data from a single explant. (A) Gustatory neurons have random growth when cultured alone. (B) When cultured with OPE, gustatory fibers grow preferentially toward the target endoderm (red vector; mean angle of 41.8°, $r = 0.422$, $P < 0.01$). (C) Growth was also directed toward the target in flank ectoderm cocultures (mean angle of 56.0°, $r = 0.503$, $P < 0.01$). (D) Growth was random with respect to GE; there was no statistically significant mean angle of eight cocultured explants. (E) Growth was also random in cocultures with NC. Scale bars, 100 μm.

is determined by multiplying the r value (denoted rj) for that explant times the cosine of the mean angle for that explant (denoted $\bar{a}j$).

$$\mathrm{Y}j = rj \sin \bar{a}j$$

$\mathrm{Y}j$ = the Y value for a single explant culture (denoted j). It is determined by multiplying the r value (denoted rj) for that explant times the sine of the mean angle for that explant (denoted $\bar{a}j$).

$$\bar{\mathrm{X}}j = \frac{rj \cos \bar{a}j}{k}$$

$\bar{\mathrm{X}}j$ = the collective X value for a set of explants ($n = 8$, denoted k)

$$\bar{\mathrm{Y}}j = \frac{rj \sin \bar{a}j}{k}$$

$\bar{\mathrm{Y}}j$ = the collective Y value for a set of explants ($n = 8$, denoted k)

$$r = \sqrt{\mathrm{X}^2 + \mathrm{Y}^2}$$

r represents the collection of fibers around the mean angle for all explants.

To test if the mean angle of axon outgrowth for a given experimental condition was significantly different from random and therefore directed, we used a parametric one-sample second order analysis of angles.

According to the test:

Ho: There is no mean population direction.
Hi: There is a mean population direction.

$$\sum \mathrm{x}^2 = \sum \mathrm{X}j^2 - (\sum \mathrm{X}j)^2/k$$

$\Sigma \mathrm{x}^2$ = the total sum of X values (see above) for all explant cultures of a given treatment minus that value divided by the total number of fibers present in that treatment (denoted k).

$$\sum \mathrm{y}^2 = \sum \mathrm{Y}j^2 - (\sum \mathrm{Y}j)^2/k$$

$\Sigma \mathrm{y}^2$ = the total sum of Y values (see above) for all explant cultures of a given treatment minus that value divided by the total number of fibers present in that treatment (denoted k).

$$\sum \mathrm{xy} = \sum \mathrm{X}j\mathrm{Y}j - (\sum \mathrm{X}j \sum \mathrm{Y}j/k)$$

$\Sigma \mathrm{xy}$ = the total sum of X and Y values for all explant cultures of a given treatment minus that value divided by the total number of fibers present in that treatment (denoted k).

These values are all used in the following formula:

$$F = \frac{k(k-2)}{2} \frac{[\bar{\mathrm{X}}^2 \sum \mathrm{y}^2 - 2\mathrm{XY} \sum \mathrm{xy} + \bar{\mathrm{Y}}^2 \sum \mathrm{x}^2]}{[\sum \mathrm{x}^2 \sum \mathrm{y}^2 - (\sum \mathrm{xy})^2]}$$

All F values were tested for significance at an alpha = 0.05 level. The critical value used for all tests was determined for a one-tailed F with 2 degrees of freedom and a k value of 8:

$$F \text{ critical}_{0.05(1),2,6} = 5.1432$$

Other statistical tests

Numerical means and standard error were determined by using Microsoft Excel. Significant differences for all measures (number of fibers exiting the explant, number of fiber

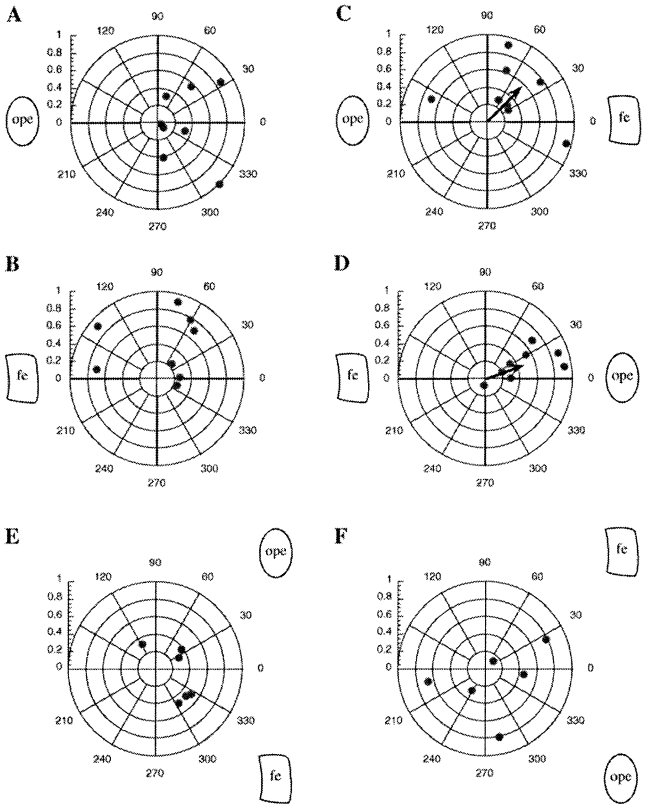

Fig. 5. Placodal neuron outgrowth is directed toward OPE or FE after 4 days in vitro, but only when the target is cocultured anterior to placodal ectoderm explants. Neither OPE (A) nor FE (B) alone was attractive to gustatory axons when these targets were placed posterior (at 180°) to the placodal ectoderm ($n = 8$). When placodal neurons were given a choice of attractive targets, one anterior at 0°/360° and one posterior at 180°, axon growth was always directed toward the anterior target. (C) Gustatory axons had a mean angle of 53.2°, ($r = 0.396$, $P < 0.05$) when FE was anterior and OPE posterior. (D) The mean angle of outgrowth for placodally derived neurites was 21.6° ($r = 0.462$, $P < 0.05$) when OPE was anterior and FE posterior. No significant mean angle was found when EP was paired with OPE and FE both anterior but at mirror 45° angles to EP. Polar plot of mean angles of six EP explants paired with (E) OPE dorsal and FE ventral, and (F) FE dorsal and OPE ventral. OPE, oropharyngeal endoderm; FE, flank ectoderm.

tips, and number of fibers contacting target) were determined by using either *t*-tests for pair-wise comparisons, or ANOVA with a *t*-method for multiple unplanned comparisons.

Results

Presumptive epibranchial placodal ectoderm contributes neurons to cranial ganglia in vivo

To confirm that our early ectoderm explants contained only the presumptive epibranchial placodes (EP), and did not contain dorsolateral placodes or cranial neural crest, which develop adjacent to epibranchial placodes (Northcutt et al., 1996), pigmented cranial ectoderm was grafted into albino hosts at stage 19 (Fig. 1A) and assayed for pigmented cells at stage 41 (hatching). The presumptive epibranchial placodal ectoderm comprises the region just lateral and ventral to the neural tube, prior to ventral movement of the presumptive dorsolateral placodes from the lateral neural folds to the subjacent ectoderm (Northcutt et al., 1996). By stage 26 (~14 h after stage 19), epibranchial placodes have thickened into columnar epithelia and then generate neurons until at least stage 35 (2 days after stage 19; Northcutt and Brändle, 1995; Stone, 1922). By stage 41 (over 7 days after stage 19), the embryos have hatched and have readily recognizable cranial ganglia (Northcutt and Brändle, 1995; Table 1). In albino animals with pigmented transplants, cells from the grafts contributed neurons to the VIIth, IXth and Xth cranial nerve ganglia, as expected (Fig. 2A–D). page As one would predict, not all the cells in the ganglia were labeled since the sensory ganglion cells are derived from both epibranchial placodes and cranial neural crest (Barlow and Northcutt, 1995; D'Amico-Martel and Noden, 1983; Narayanan and Narayanan, 1980). Labeled cells were not detected in receptor cells (e.g., neuromasts and ampullary organs; data not shown) or in ganglia of the lateral line system (Fig. 2C; gST), both of which are derived from the dorsolateral placodes (Northcutt and Brändle, 1995; Northcutt et al., 1995). Further, labeled cells were never detected in the trigeminal ganglion (Fig. 2E and F), which does not receive cells from the epibranchial placodes (see Webb and Noden, 1993). The neurons that arise from ectodermal grafts are derived from the epibranchial placodes, and represent an embryonically homogeneous subset of cranial ganglion viscerosensory neurons.

Explanted preplacodal ectoderm gives rise to neurons in vitro

Although the grafted presumptive epibranchial placodes differentiated normally in vivo, contributing neurons to the appropriate ganglia, it was critical to determine whether these presumptive placodes were also specified at this early stage, and therefore would make neurons in vitro. When presumptive epibranchial placodal ectoderm was explanted at stage 19 and raised in GFR-Matrigel, this tissue gave rise to differentiated neurons with extensive neuritic processes (Fig. 3A and B), which were immunopositive for the neural marker, acetylated alpha-tubulin (anti-AT; Fig. 3A). These data indicate that, by stage 19, prior to the formation of placodal thickening beginning at stage 26 (Northcutt and Brändle, 1995; Stone, 1922), the region destined to give rise to epibranchial placodes is already specified, and can give rise to differentiated neurons in the absence of additional signals.

Presumptive placodal ectoderm from stage 19 embryos was cultured for 4, 6, or 8 days. These time points correspond loosely to when gustatory fibers first arrive at the pharyngeal epithelium, first make contacts with undifferen-

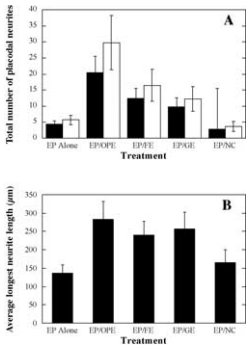

Fig. 6. OPE enhances quantitative aspects of gustatory axon outgrowth. (A) Placodal neurons cultured with OPE for 4 days have significantly more neurites exiting each placodal ectoderm explant than placodal neurons cultured alone or with NC (*, $P < 0.01$). Outgrowth in cocultures with nonattractive targets—GE or NC, or with attractive FE, does not differ from that of placodal neurons cultured alone or from each other. (B) OPE cocultures also have more fiber tips than EP explants grown alone or with NC (**, $P < 0.05$). EP, epibranchial placodes; OPE, oropharyngeal endoderm; FE, flank ectoderm; GE, gut endoderm; NC, notochord; $n = 8$, all treatments.

tiated taste receptor cells, and likely make synaptic contacts with taste buds in vivo, respectively (Barlow et al., 1996). We found that placodal development in vitro approximates the timing of cranial nerve development in vivo (Table 2; Northcutt and Brändle, 1995). After 4 days in culture, the average length of the five longest fibers was approximately 200 μm, the average number of fibers exiting the explant was 8.4 $\pm$ 3.4, and the average number of fiber tips per culture was 18.3 $\pm$ 4.4. These values continue to increase through 6 and 8 days in vitro (Table 2), indicating that extending the duration of the culture period to 8 days did not arrest development of placodal neurons, but rather fiber number and length continued to increase.

The pattern of early growth of placodal fibers under control and co-culture conditions

Having ascertained that cultured presumptive placodal ectoderm will generate neurons which send out axons, we next chose to better characterize and quantify neurite outgrowth when placodes were raised alone or in coculture with various targets. First, the distribution of placodal fibers was determined by counting the number of cultured neurites that fell within each of 12 30° arcs of a 360° circle around each placodal explant (Fig. 3C). Initially, 2 approaches were used to tally fiber distribution: (1) the number of fiber tips in each of the 12 30° segments was counted (Fig. 3C, gray circles); and (2) the number of fibers exiting the explant in each of the 12 30° segments was documented (Fig. 3C, black triangles). Because there was no statistical difference between the 2 values (data not shown), all data presented here are from the assessment of the number of fiber tips in each of the 12 30° segments. Additionally, each fiber tip was assigned a radial value on a polar plot of each explant. The average direction, or mean angle, of fiber growth for all axons of a single placodal explant was calculated by using circular distribution statistics (see Materials and methods for details). A second order analysis of the mean angle of outgrowth for all cultures within a particular treatment was then used to determine whether neurite growth was directed or random within a treatment (Fig. 3D).

When presumptive placodal ectoderm was grown alone, fiber outgrowth was randomly distributed around the explant (Figs. 3 and 4A). However, when oropharyngeal endoderm (OPE) explants were cultured with the placodal explants, the fibers grew preferentially toward the OPE (red arrow; $P < 0.001$; Fig. 4B). Surprisingly, placodally derived fibers also grew toward flank ectoderm (FE) explants (red arrow; $P < 0.001$; Fig. 4C), which had been selected initially to serve as a nonattractive target control for axon outgrowth. Nonetheless, growth of EP neurites toward FE or OPE was statistically indistinguishable (data not shown). These results suggested that early placodal neurons might be attracted toward any embryonic tissue, and thus the directional growth effect observed was nonspecific. To test this idea, placodal explants were paired with two other types of tissue that placodal fibers do not encounter in vivo: gut endoderm (GE), and a portion of the notochord (NC). Gustatory fiber growth was random with respect to both of these tissues (Fig. 4D and E); these test targets were not attractive. We concluded that only OPE and FE were specifically attractive to placodal neurons over a range of a few hundred microns (average = 350 μm), during the phase of development when these axons are finding their target epithelium in vivo.

Orientation of placodal ectoderm with respect to the target is crucial for directed growth

Next, we tested whether the orientation of the placodal explant with respect to target tissue was important for the observed, long-range chemoattractive effect. All 4-day culture experiments described above entailed placing the cocultured target explant anterior to the placodal explant, i.e., at 0°/360°. To determine whether fiber growth toward OPE and FE was dependent on the position of the target relative to the placodal ectoderm, we altered the positions of the tissues in culture. Targets were placed posterior to the placodal explant, at 180°, rather than anteriorly at 0°/360°. In this configuration, growth was random with respect to either OPE and FE (Fig. 5A and B; compare with Fig. 4B and C). In a second set of experiments, placodal neurons were confronted with both OPE and FE in an anterior/posterior choice paradigm. When OPE was placed anterior and FE posterior to placodal explants, neurites grew toward the OPE (Fig. 5C). When the positions of the targets were reversed, and FE was placed anteriorly, axon outgrowth was now directed toward FE (Fig. 5D). Further, when OPE was placed posteriorly and was paired with anteriorly placed NC, placodal fibers outgrowth was again random (data not

shown). Thus, placodal fiber growth is only directed toward an attractive target explant when these targets are placed anterior to placodal ectoderm explants, reflecting an unexpected intrinsic bias of the EP axons revealed only in the presence of attractive targets.

We next asked whether, given a choice of targets placed at mirrored 45° angles anterior to the EP explant, a preference of placodal fibers for OPE emerged. Although growth tended to be biased in an anterior direction, there was no significant orientation of fibers toward one or the other target regardless of which one was placed dorsal versus ventral with respect to the EP explant (Fig. 5E and F). Thus, either EP axons cannot distinguish between OPE and FE at this stage, or our measure of axon directedness is not refined enough to uncover a preference for one target over the other.

Target type influences gustatory fiber number in vitro

Although both FE and OPE were attractive to placodal neurites, only OPE substantially enhanced neurite outgrowth via two measures made at 4 days in vitro (DIV). The total number of fibers exiting each placodal explant in OPE cocultures was significantly greater than in placodal cocultures with notochord, or placodes alone (Fig. 6A; treatment effect via ANOVA, $P < 0.002$; with *t*-method for multiple comparisons, $P < 0.01$). Similarly, the number of fiber tips in EP/OPE cocultures was significantly greater than in the control condition, or in cocultures with notochord (Fig. 6B; ANOVA, $P < 0.005$; *t*-method for multiple comparisons, $P < 0.05$). Placodal explants cocultured with FE or gut endoderm were not different from controls, nor did they differ from OPE cocultures.

In an attempt to discern if the observed enhancement of placodal neurite outgrowth was due to an increase in branching triggered by OPE, we examined the ratio of the number of fiber tips to the number of fiber exits. While both of these measures were increased only in OPE cocultures, their ratio did not differ significantly from other treatments (data not shown), indicating that the tendency to branch was similar regardless of the type of coculture.

The trophic effect of OPE on placodal outgrowth was observed regardless of the location of OPE with respect to the placodal explant, or the presence or absence of a second target. In the presence of OPE, the number of fibers exiting and the number of fiber tips was significantly greater than when placodes were cultured alone, but there was no statistical difference among treatments via ANOVA analysis (data not shown).

Only OPE supports long term growth and survival of placodal neurons

In the next phase of this study, the culture period was extended to assess placodal neurite behavior at progressive stages when, in vivo, neurites likely encounter undifferentiated taste buds [6 days in vitro (DIV)], make synaptic contacts with differentiated taste buds (8 DIV), and when axolotl larvae have begun to feed (10 DIV; Bordzilovskaya et al., 1989).

At the 6 day time point, there is no significant difference in axon outgrowth of placodal explants cultured with FE versus those grown with OPE (Figs. 7A, 8A and B, and 9). By 8 DIV, however, the number of fibers exiting placodal explants is statistically greater in OPE cocultures compared with FE cocultures, as is the number of fibers contacting target explants (Figs. 7B, 8C and D, and 9). The difference in axon outgrowth between OPE and FE cultures increases by 10 DIV. The number of fibers exiting and the number of fiber tips are dramatically greater in OPE cocultures. This difference is attributable in part to an increase in growth from 8 DIV in OPE cocultures, but also to a reduction in neuronal outgrowth in EP/FE cocultures (Fig. 9A and B). Further, substantial numbers of fibers approached and invaded the OPE explants (Figs. 8E, 9 and 10). In contrast, few if any fibers were present in FE cocultures, and only rare fibers even approached FE explants (Figs. 8F and 9).

Discussion

We have devised a new approach to study the interaction of a relatively homogeneous population of developing viscerosensory neurons with taste bud-bearing oropharyngeal epithelium. By culturing the embryonic fields fated to give rise to these tissues long before sensory neurons and taste buds differentiate, we can examine and manipulate the entire process of taste epithelium innervation in vitro. This includes the genesis and differentiation of sensory neurons, including gustatory neurons, and their taste bud targets, as well as axon outgrowth, guidance, and contact with and trophic support by differentiated target oropharyngeal endoderm. Here, we show that cultured presumptive placodal ectoderm will generate new sensory neurons which send out neurites with a time course comparable to those of neurons in intact embryos (Barlow et al., 1996; Northcutt and Brändle, 1995). While placodal neurons cultured alone have random axonal outgrowth, those cultured with oropharyngeal endoderm, and surprisingly flank ectoderm, direct their neurites toward these targets in the short term. In longer term experiments, however, oropharyngeal endoderm alone promotes gustatory neuron development, likely providing trophic support and perhaps neurogenic cues.

Most studies examining axon guidance in the peripheral nervous system in culture rely on excised immature ganglia as a source for sensory neurons (Kobayashi et al., 1997; Lumsden and Davies, 1983; Luukko et al., 1998; Rochlin and Farbman, 1998; Rochlin et al., 2000; Tashiro et al., 2000). One advantage of this approach is that neuronal phenotype is typically already established at the time of explantation. However, a disadvantage is that neuronal development is interrupted when differentiated neurons are axotomized during removal. Whether this surgery affects

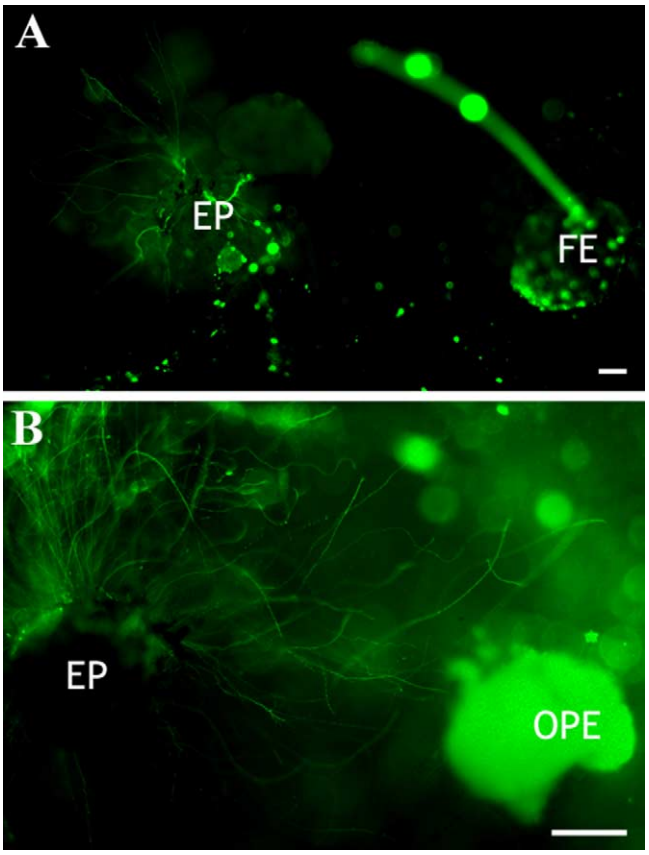

Fig. 7. Fluorescence micrographs of EP cocultures. (A) EP explant paired with FE target ectoderm, fixed and immunostained with anti alpha-acetylated tubulin (anti-AT; green), after 6 days in vitro. Fibers can be seen extending from the EP explant. Anti-AT also recognizes cilia of the embryonic FE (green dots in FE). (B) Whole-mount view of an EP explant paired with an OPE target, immunostained after 8 days in vitro. Numerous fine fibers are apparent. Due to a large amount of autofluorescent yolk granules, the OPE explant is highly fluorescent. In both (A) and (B), many fibers are not detected since they are located out of the focal plane of the micrographs. EP, epibranchial placodes; OPE, oropharyngeal endoderm; FE, flank ectoderm. Scale bars in (A) and (B), 200 μm.

subsequent development has not been tested. Transient exposure of cultured sensory neurons to BDNF can hasten the onset of neuronal reliance on this neurotrophin (Vogel and Davies, 1991), indicating that early neuronal experience impacts subsequent neuronal behavior. In addition, in the developing spinal cord, once commissural axons have encountered floor plate cells at the midline, they are now recalcitrant to chemoattractant signaling (Shirasaki et al., 1998), and become newly sensitive to local repellant cues (Zou et al., 2000) that prevent axons from recrossing the midline (Kaprielian et al., 2001, for review). Thus, in studies where early ganglia are explanted and their outgrowth assessed, immature axons have already sampled the local environment prior to removal, and this experience may alter the subsequent development of these sensory neurons. In contrast, the culture system we have developed permits de novo genesis of neurons which generate new and naïve neurites.

An additional advantage of our system is the relative homogeneity of the cultured sensory neurons. Cranial nerve ganglion cells have two embryonic sources: the cranial neural crest and epibranchial placodes (Stone, 1922; Yntema, 1937, 1943; Hörstadius, 1950; Narayanan and Narayanan, 1980; D'Amico-Martel and Noden, 1983; Northcutt and Brändle, 1995; Chai et al., 2000). Neurons derived from each of these embryonic tissues arise in locations remote from one another, and through migration and morphogenesis, are joined together into various cranial nerve ganglia (Begbie and Graham, 2001). Though not definitively known, it is thought that neural crest-derived neurons are somatosensory, while placodal neurons give rise to viscerosensory neurons, including gustatory neurons which innervate taste buds and carry taste information (Landacre, 1910; Baker and Bronner-Fraser, 2001, for review). Whereas explantation of embryonic ganglia for in vitro studies results in the culture of a spectrum of neuronal types, culturing presumptive epibranchial placodes, prior to neurogenesis and long before ganglion formation, allows us to obtain a more homogeneous population of presumed gustatory neurons.

One potential pitfall in our approach is that explanted preplacodal ectoderm, while generating neurons in vitro, may give rise to cells that do not differentiate as they would have in vivo. Experiments examining the degree of determination of the ophthalmic placode of the trigeminal ganglion indicate that preplacodal ectoderm grafted to ectopic, albeit permissive, sites on the trunk gives rise to neurons that display numerous characteristics of trigeminal neurons despite their foreign location in trunk or ectopic ganglia (Baker et al., 2002). Similarly, explanted preplacodal epibranchial ectoderm produces small numbers of neurons in vitro, even without further induction by pharyngeal endoderm, and these cultured neurons express the transcription factor *Phox2a* that would normally mark their appropriate development in vivo (Begbie et al., 1999). Results of our studies are consistent with, but not definitive for, specification of epibranchial placodal ectoderm prior to formation of morphologically distinct placodes. First, we have shown that the preplacodal cranial ectoderm gives rise to the correct neurons when grafted in vivo. Second, neurons generated from this ectoderm in vitro respond with enhanced growth to their presumed proper target, the oropharyngeal endoderm. A concise answer to the issue of placodal neuron specification awaits a more complete morphological and molecular characterization of the development of epibranchial placodal neurons.

Target oropharyngeal endoderm is chemoattractive and provides trophic support to gustatory neurons in vitro during the time these neurons pathfind to the taste epithelium in vivo

Epibranchial placodes form by stage 25 in axolotls, and over the next 2 days generate immature neurons. Subse-

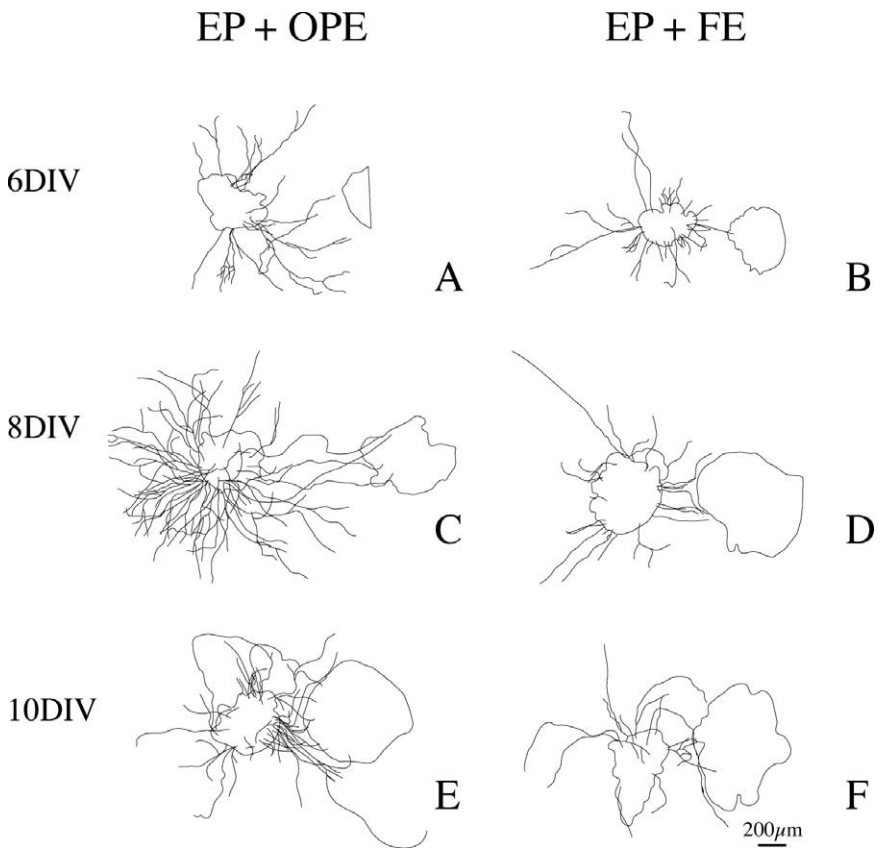

Fig. 8. Under long term culture conditions, only OPE supports the continued growth and maintenance of EP fibers, whereas neurites regress in EP/FE cocultures. (A, B) Representative racings of acetylated alpha-tubulin-immunopositive fibers in cocultures of placodal tissue with OPE (A, C, E) and FE (B, D, F), at 6 DIV (A, B), 8 DIV (C, D), and 10 DIV (E, F). Scale bar, 200 μm.

quently, the placodes regress, and the new sensory neurons begin to send out axons which reach the oropharyngeal epithelium 2 days later at stage 37 (Table 1; Barlow et al., 1996; Northcutt and Brändle, 1995). The time course of epibranchial placodal neuron development in vitro is comparable. After 4 DIV or the equivalent of stage 37, neurons have formed and sent out axons. This early placodal neurite outgrowth is directed toward the appropriate target tissue, the oropharyngeal endoderm, indicating that early on, this target tissue releases long range, diffusible cues which attract gustatory fibers. Further, the early chemoattractiveness of oropharyngeal endoderm is moderately specific. Neither notochord nor gut endoderm explants elicited directed growth. However, flank ectoderm was equally attractive to placodal neurons during this first phase of neurite development. These data indicate that embryonic tissue is not broadly permissive for axon outgrowth, and suggest either that: (1) oropharyngeal endoderm and flank ectoderm both possess the same chemical cue(s) that attracts placodal neurons; or (2) placodal neurons are competent to respond to two different chemoattractants—one produced by the endoderm, another by the ectoderm.

One important caveat is that the interaction between sensory neurons and their targets in vitro does not mirror the process of axon guidance in vivo. The diffusion of target-derived secreted signals is likely greater in vitro than in vivo, so that diffusible factors act over a greater distance under gel culture conditions (see Rochlin et al., 2000). That gustatory neurons can respond to the appropriate target in culture indicates that the interaction is biologically relevant, but the anatomical context for this signaling is clearly disrupted. Therefore, we cannot yet distinguish between two possibilities for the role of target derived diffusible factors in vivo. One possibility is that the OPE possesses an attractive cue that is similar to that emitted by the cranial mesenchyme encountered by developing gustatory axons as they grow toward the epithelium. Alternatively, the OPE is actually attractive from a distance in vivo.

Several molecules have been identified recently that guide developing axons from a distance, and many of these factors are expressed in the developing taste periphery. The secreted semaphorin, Sema3a, is expressed in the developing tongue of rat embryos (Giger et al., 1996), and both tongue explants and Sema3A repulse early axon outgrowth from cultured trigeminal and geniculate sensory neurons (Rochlin and Farbman, 1998; Rochlin et al., 2000). Despite this repulsion, branches of each of these nerves do innervate the tongue, so that additional signals must attract these neurites initially. The role of Sema3a in the tongue appears to be to control the gradual progression

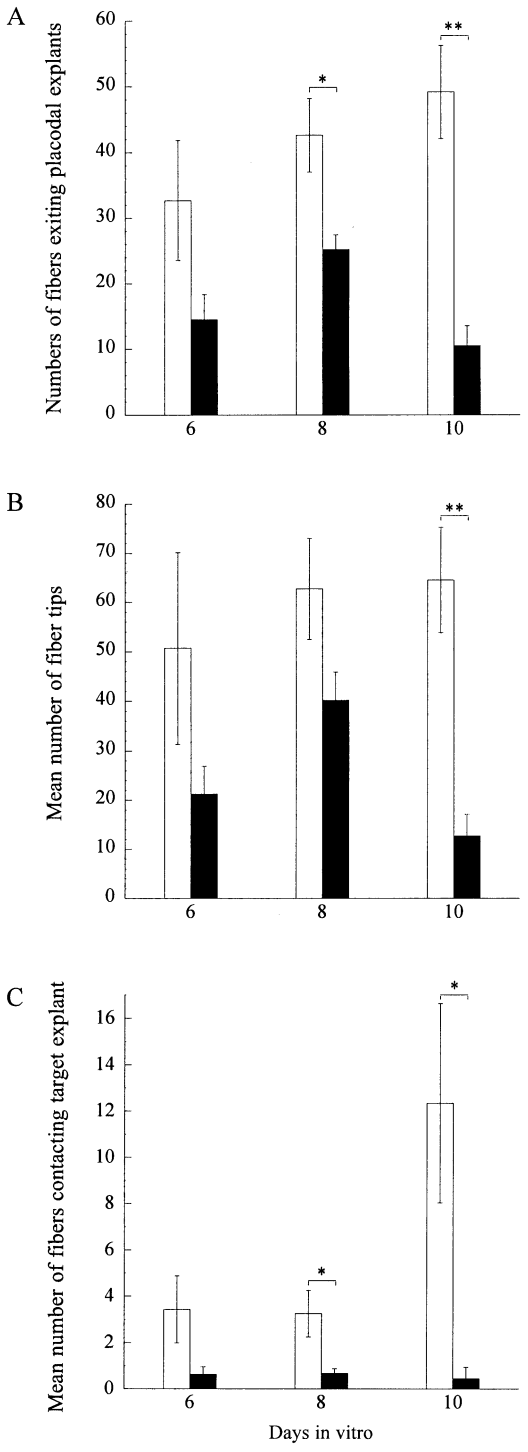

Fig. 9. Quantification of axon outgrowth with respect to OPE and FE with increasing culture duration. (A) Mean number of fibers exiting placodal explants in OPE cocultures (white bars) is significantly greater than in FE cocultures (black bars) beginning at 8 DIV ($P < 0.05$ 8 DIV; $P < 0.001$ 10DIV). (B) At 6 and 8 DIV, the mean number of fiber tips in OPE (white bars) versus FE (black bars) cocultures is not different, whereas by 10 DIV, placodal explants paired with OPE have significantly more fiber tips ($P < 0.001$). (C) The mean number of fibers contacting target explants is initially quite low, and not different between coculture types. By 8 DIV, significantly more fibers contact OPE targets (white bars) than FE targets (black bars), and this difference is increased by 10 DIV ($P < 0.05$). *, $P < 0.05$; **, $P < 0.001$.

of innervation first to lateral domains of the tongue and eventually to allow fibers to invade the lingual midline (Rochlin and Farbman, 1998).

Neurotrophins have been implicated as guidance factors, in addition to their role as classic neural support factors, and are also expressed in the developing tongue. Trigeminal neurons grow preferentially toward maxillary process explants in vitro (Lumsden and Davies, 1983), and recently the attractive factors have been identified as a combination of Neurotrophin-3 (NT-3) and Brain-Derived Neurotrophic Factor (BDNF) (O'Connor and Tessier-Lavigne, 1999). Significantly, both NT-3 and BDNF are expressed in the developing lingual epithelium, and onset of expression occurs prior to the arrival of sensory nerves fibers (Nosrat et al., 1996; Nosrat and Olson, 1995). Thus, these neurotrophic factors may be as short range guidance cues in the taste periphery.

Finally, the secreted factor, Sonic Hedgehog (SHH), has been shown recently to act in axon guidance (Charron et al., 2003). While SHH is expressed in the developing tongues of mice and rats (Bitgood and McMahon, 1995; Hall et al., 1999), SHH is unlikely to be responsible for the long range chemoattractiveness of the oropharyngeal endoderm in our cultures. SHH is highly expressed in the developing notochord (mouse: Echelard et al., 1993; zebrafish: Krauss et al., 1993; axolotl: L.B., unpublished observations); nonetheless, the notochord is completely nonattractive to placodal neurons.

Placodal neurons display a bias toward anterior growth

One unanticipated finding from this study was that placodal axons grew toward OPE or FE, but only when these targets were placed anterior to placodal ectoderm explants. These results imply that inherent guidance cues are present in the nonplacodal ectoderm. The epibranchial placodes themselves occupy a relatively small region of each ectodermal explant (Stone, 1922; Northcutt and Brändle, 1995; Schlosser and Northcutt, 2000), with the remainder of the ectoderm destined to differentiate as surface epithelium (Barlow and Northcutt, 1995; Northcutt et al., 1996). There has been some suggestion that this ectoderm is patterned quite early and possesses axial information by neurula stages (Couly and LeDouarin, 1990). Thus, positional cues may be present within the ectodermal explant for the developing placodal neurons. For example, extracellular matrix components, such as laminin, have been implicated in permissive axon growth (Moody et al., 1989; Tisay and Key, 1999). One explanation for the anterior propensity of placodal fibers would be that permissive proteins are present in the anterior region of the ectodermal explant, and not the posterior. However, this scenario seems an oversimplification, given that gustatory axon outgrowth is random when ectoderm containing placodes is grown alone. If positional cues present in ectoderm were sufficient to guide placodal neurons, then axon trajectories should always be in the

anterior direction, regardless of the presence or absence of appropriate target explants. A more plausible explanation is that gustatory axon outgrowth is directed toward anterior targets via a combination of cues, including permissive, ectoderm-derived signals, and longer range chemoattractants, perhaps from the oropharyngeal endoderm.

Only the appropriate target, oropharyngeal endoderm, provides trophic support for placodal neurons

While both flank ectoderm and oropharyngeal endoderm are equally attractive to early gustatory neurons, a clear distinction in overall neuronal growth with respect to target type is evident. In general, placodal neurons have significantly more axonal outgrowth when paired with OPE both in the short and long term. In particular, in prolonged cultures, the specificity of the interaction between gustatory neurons and OPE becomes distinct from axonal behavior with respect to FE. Only OPE supports the axons of these neurons in the long term, as evidenced by the exuberant growth in 10-day cultures. However, we cannot discern the precise effect(s) of the target tissue on gustatory neurons in the long term. Several reasonable hypotheses can be suggested, and none are mutually exclusive.

One possibility is that OPE may continue to induce the formation of more neurons from placodal ectoderm, as pharyngeal endoderm is a known inducer of epibranchial placodes (Begbie et al., 1999). However, by stage 35 (or the equivalent of 2 days in vitro), placodes have regressed and thus no longer generate new neurons (Northcutt and Brändle, 1995). Placodes likely also cease to generate neurons in vitro after a few days, and thus the increasing differences between OPE and FE in older cocultures cannot be explained by this mechanism. Interestingly, recent evidence indicates that neurogenesis continues within developing epibranchial ganglia, in that the these placodes give rise to mitotically active daughter cells (Begbie et al., 2002). This additional neurogenesis also may be governed by signals from oropharyngeal endoderm, and could explain enhanced axonal development in EP/OPE cocultures in the long term.

Second, rather than increasing neuronal number, coculture with OPE may induce branching of gustatory axons. For example, contact with target Merkel cells increases branching of trigeminal axons in vitro (Vos et al., 1991), and branching patterns of ciliary ganglion axons are also affected by contact with target muscle cells (Berman et al., 1993). In our studies, the number of fiber tips is typically greater than the number of fibers exiting placodal explants, which implied that these fibers are branching in vitro. However, when we examined the ratio of exiting fibers to fiber tips, the ratio did not differ significantly among coculture treatments, implying that differences in branching are not likely to be responsible for enhanced axonal growth in OPE cocultures.

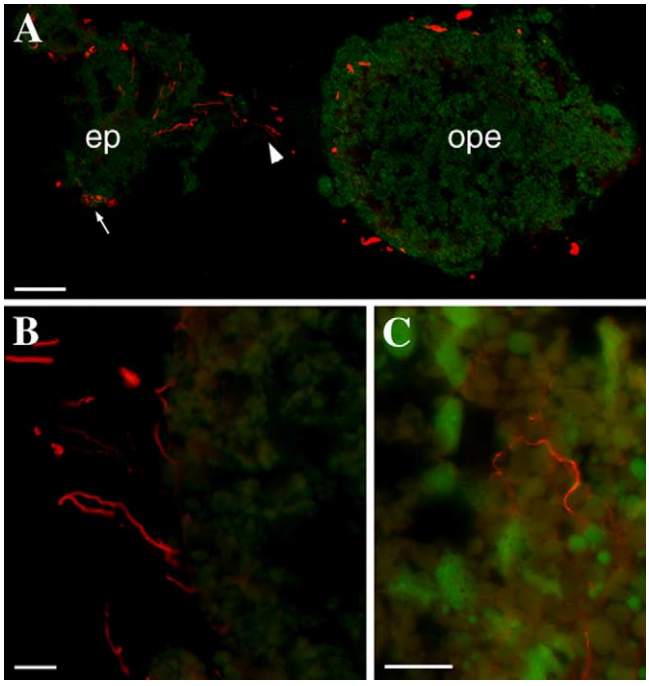

Fig. 10. Placodal fibers invade OPE explants under long term culture. (A) A low magnification image of a cryosection through paired explants immunostained for alpha-acetylated tubulin (red). Immunoreactive neuronal cell bodies are present (arrow) as are sections of nerve fibers (arrowhead) coursing toward the target OPE. At higher magnification, fine neurites are seen at (B) the OPE surface, and (C) deep within the explant. In (A) and (B), autofluorescent yolk granules are in green and are used to delineate the extent of the explants. In (C), Hoechst counterstained nuclei have been digitally altered to appear green, while the smaller yolk granules autofluoresce in both channels. ope, oropharyngeal endoderm; ep, epibranchial placodes. Scale bars: (A), 100 μm; (B and C), 10 μm.

A final explanation for the presence of large numbers of fibers in OPE cocultures is that this target offers neurotrophic support to gustatory neurons, whereas FE fails to do so. Gustatory neurons paired with FE have lost most if not all axons, and may have undergone cell death. This outcome would be predicted given the classic neurotrophic hypothesis, that neurons must obtain sufficient neurotrophic support from appropriate target cells in order to survive (Oppenheim, 1989); FE, while possessing an early chemoattractant, does not produce the appropriate neurotrophic molecules, and thus gustatory neurons are not supported. Both BDNF and NT-3 are expressed in the developing and mature taste epithelium of mammals (Nosrat et al., 1996, 2000; Nosrat and Olson, 1995), and subsets of cranial nerve ganglion cells are greatly reduced in mice that are homozygous null for either of these neurotrophins or their receptors (Conover et al., 1995; Ernfors et al., 1994; Jones et al., 1994; Liebl et al., 1997; Silos-Santiago et al., 1997; Zhang et al., 1997). Therefore, neurotrophins within the target OPE may be supporting placodal neurons, whereas the necessary neurotrophin complement is not present in FE explants.

A dynamic interaction between pharyngeal endoderm and placodally derived gustatory neurons occurs throughout embryonic development

Little is known about the details of genesis of sensory neurons from epibranchial placodes. However, these ectodermal thickenings are induced and produce neurons in response to signals emitted by the oropharyngeal endoderm (Begbie et al., 1999; S. Matz and R.G. Northcutt, personal communication). More precisely, BMP7 is expressed by pharyngeal endoderm, and induces, from a distance, neurogenesis in cultured epibranchial placodal ectoderm (Begbie et al., 1999). Our results indicate that the interaction between pharyngeal endoderm and placodal neurons persists as development progresses; OPE attracts placodally derived sensory neurons from a distance, and then in the long term, provides trophic support for these cells. It is also possible that BMP7 or other factors secreted by the OPE continue to induce the formation of placodal neurons. Most recently, epibranchial placodes have been found to give rise to mitotically active neuronal precursor cells, which presumably go on to generate sensory neurons (Begbie et al., 2002). OPE may also impact this aspect of epibranchial placodal neurogenesis. In sum, it is apparent that there is an important, prolonged, and dynamic interaction between epibranchial placodes, their daughter neurons and their target, the oropharyngeal endoderm.

Acknowledgments

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